Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical and Biophysical Research Communications 338 (2005) 1860–1864
BBRC
www.elsevier.com/locate/ybbrc

Identification of tick periviscerokinin, the first neurohormone of Ixodidae: Single cell analysis by means of MALDI-TOF/TOF mass spectrometry

Susanne Neupert [a,b,*], Reinhard Predel [a,b], William K. Russell [c], Ronald Davies [d], Patricia V. Pietrantonio [e], Ronald J. Nachman [b]

[a] Saxon Academy of Sciences, Research Group Jena, Erbertstrasse 1, 07743 Jena, Germany
[b] Areawide Pest Management Research, Southern Plains Agricultural Research Center, USDA, 2881 F/B Road, College Station, TX 77845, USA
[c] The Laboratory for Biological Mass Spectrometry, Department of Chemistry, Texas A&M University, College Station, TX 77843, USA
[d] Cattle Fever Tick Research Laboratory, USDA-ARS, 22675 N. Moorefield Road, Edinburgh, TX 78541, USA
[e] Department of Entomology, Texas A&M University, College Station, TX 77843, USA

Received 20 October 2005
Available online 9 November 2005

Abstract

The first peptidergic neurohormone from the ticks *Ixodes ricinus* and *Boophilus microplus* has been identified by using a combination of immunocytochemistry and mass spectrometric analysis of single cells. The novel peptide (Ixori-PVK, PALIPFPRV-NH_2) shows a high sequence homology with members of the insect periviscerokinin/$CAP2_b$ peptides that in insects are involved in the regulation of water balance. The function of this peptide in ticks is still unknown, but these pests consume large amounts of blood in a single blood meal which is a challenge for the regulation of diuretic processes. Thus, the novel peptide may be involved in one of the key physiological processes in ticks. High energy collision-induced dissociation was successfully used to distinguish between Leu/Ile ambiguities in single cell preparations. This is the first successful de novo sequencing of a peptide from single cell preparations of arthropods.

Keywords: MALDI-TOF/TOF mass spectrometry; Neuropeptide; Periviscerokinin; *Boophilus*; *Ixodes*; Tick; Single cell analysis

Members of the family Ixodidae, commonly known as ticks, cause great economic losses of livestock; an estimated damage of several million dollars per year is reported for the United States [1]. As obligatory blood feeders, ticks consume large amounts of blood in a single blood meal which is a challenge for the regulation of diuretic processes. The necessity to remove excessive water after a blood meal has also been observed in a number of insects such as mosquitos [2–4], blood-feeding bugs (e.g., *Rhodnius prolixus*) [5,6], fleas [7], and lice [8]. In the insects, diuresis is known to be regulated by several neuropeptides which influence secretion and/or reabsorption processes in the Malpighian tubules and the rectum [9]. Among these neuropeptides, are the insect kinins and perviscerokinin/$CAP2_b$-related peptides which may occur with multiple forms in a single species [10–14]. However, nothing is known about the hormonal control of excretion in ticks and no neuropeptides have been unambiguously identified from any tick so far. Recently, a receptor with sequence similarity to an insect kinin receptor (*Drosophila melanogaster*, CG 10626) [15] has been identified from the Southern Cattle Fever tick *Boophilus microplus* (CG10626) [16] and shown to functionally respond to various kinin peptides [17].

In this study, we searched for peptidergic neurohormones that may influence the water balance of ticks by screening the nervous system for peptide homologs to the insect kinins and periviscerokinin/$CAP2_b$-related peptides. Antisera against these neuropeptides revealed the expression of immunoreactive substances in a few neurons. We

* Corresponding author. Fax: +49 3641 949192.
E-mail address: mail@susanne-neupert.de (S. Neupert).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.10.165

Report Documentation Page

Form Approved
OMB No. 0704-0188

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE **OCT 2005**	2. REPORT TYPE	3. DATES COVERED **00-00-2005 to 00-00-2005**

4. TITLE AND SUBTITLE **Identification of tick periviscerokinin, the first neurohormone of Ixodidae: Single cell analysis by means of MALDI-TOF/TOF mass spectrometry**	5a. CONTRACT NUMBER 5b. GRANT NUMBER 5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER 5e. TASK NUMBER 5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U.S. Department of Agriculture,Areawide Pest Management Research,2881 F/B Road,College Station,TX,77845**	8. PERFORMING ORGANIZATION REPORT NUMBER
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S) 11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
see report

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT **unclassified**	b. ABSTRACT **unclassified**	c. THIS PAGE **unclassified**	**Same as Report (SAR)**	**5**	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

used the advantages of MALDI-TOF/TOF mass spectrometry to perform an analysis of those neurons that were found to contain immunoreactive material. This strategy resulted in the identification of a tick neuropeptide, which is a member of the periviserokinins, a peptide family that had been found only in insects so far. High energy collision-induced dissociation was used to distinguish between Leu/Ile in single cell preparations. As shown in our study, the novel peptide is expressed in another tick as well.

Materials and methods

Animals. Southern Cattle Fever ticks (*B. microplus*) were maintained at the Cattle Fever Tick Research Laboratory (USDA-ARS, Mission, TX), where all preparations on this pest were performed as well. The *Ixodes ricinus* was caught in Jena (Germany) by sweeping a net in grass fields. Only unfed adult ticks were used throughout the experiments.

Dissection and sample preparation for mass spectrometry. Isolated central nervous system tissue of adult ticks was fixed with microneedles in a chamber containing insect saline. A number of putative neurosecretory neurons could be identified due to their slight bluish color (Tyndall effect). After selection of the neurons of interest, the ganglionic sheath was partially removed and the respective neurons were individualized. Without any enzyme treatment, cells were removed step by step using an uncoated glass capillary and transferred to a stainless steel sample plate for MALDI-TOF mass spectrometry. Subsequent sample preparation was performed as described in Neupert and Predel [18].

MALDI-TOF/TOF tandem mass spectrometry. MALDI analysis was performed on the ABI 4700 proteomics analyzer (Applied Biosystems, Framingham, MA). Due to the nature of the samples all acquisitions were taken in manual mode. Initially the instrument was operated in reflectron mode, in order to determine the parent masses. A laser intensity of 3800 was typically employed for ionizing the neuropeptides. For the tandem MS experiments, the CID acceleration was 1 kV in all cases. The number of laser shots used to obtain a spectrum varied from 500 to 5000, depending on signal quality. In order to change the net amount of activation energy imparted to the primary ions, the collision gas (atmospheric air) pressure was increased. Three gas pressures were employed: none, medium, and high. The fragmentation patterns from these three different settings were used to determine the sequence of the peptide. The fragmentation data obtained in these experiments was handled using the Data ExplorerT software package.

Immunocytochemistry. Dissected nervous system was fixed for 24 h at 4 °C with 4% formaldehyde in phosphate-buffered saline (PBS), pH 7.2. Subsequently, preparations were washed in PBS–4% Triton X-100 and PBS–1% Triton X-100 for 24 h, respectively. Then, the preparations were incubated for 3 days at 4 °C in anti-Pea-PVK-2 serum [19] (1:4000) diluted with PBS–1% Triton X-100 containing 0.25% bovine serum albumin and normal goat serum. Following washing in 0.1 mol/L Tris–HCl, 3% NaCl, and 1% Triton X-100 (pH 7.6) for 24 h, the Fluorochrome-labelled (Cy3; Amersham, Braunschweig, Germany) secondary antibodies were directly used as a mixture in PBS–bovine serum albumin (2.5 mg/ml) at a concentration of 1:3000 for 3 days. The preparations were washed again for 24 h in 0.1 mol/L Tris–HCl, 3% NaCl, 1% Triton X-100 (pH 7.6), and then incubated at 4 °C in anti-leucokinin-1 (DPAFNSWGa) serum (1:4000) diluted with PBS–1% Triton X-100 containing 0.25% bovine serum albumin and normal goat serum. After 3 days, the samples were washed (24 h) in 0.1 mol/L Tris–HCl, 3% NaCl, and 1% Triton X-100 (pH 7.6), the Fluorochrome-labelled secondary antibodies Cy2 were directly used as a mixture in PBS–bovine serum albumin (2.5 mg/ml) at a concentration of 1:3000 for 3 days. Finally, the preparations were washed again 24 h in 0.1 mol/L Tris–HCl, 3% NaCl, and 1% Triton X-100 (pH 7.6) and transferred to glycerol. For visualization, tissues were dehydrated in ethanol, cleared in methyl salicylate, and mounted in entellan (Euromex microscopes Holland).

Documentation. Immunostainings were examined with a confocal laser scanning microscope (ZEISS LSM 510 Meta system, Jena, Germany), equipped with a HeliumNeon1 laser (wavelength 543 nm) and an Argon2 laser (wavelength 458, 477, 488, and 514 nm). Serial optical sections were assembled into combined images. Images were exported and processed with Adobe Photoshop 7.0 software.

Peptide synthesis. Ixori-PVK (PALIPFPRV-NH_2) was synthesized via Fmoc methodology on Rink Amide resin (Novabiochem, San Diego, CA) using Fmoc protected amino acids (Advanced Chemtech, Louisville, KY) on an ABI 433A peptide synthesizer (Applied Biosystems, Foster City, CA) under previously described conditions [20]. The crude product was purified on a Waters C_{18} Sep Pak cartridge and a Delta-Pak C_{18} reverse-phase column (8 × 100 mm, 15 μm particle size, and 100 Å pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: initial solvent consisting of 20% B was followed by the Waters linear program to 100% B over 40 min; flow rate, 2 ml/min. Delta-Pak C-18 retention time: Ixori-PVK (PALIPFPRV-NH_2), 9.0 min. The peptide was further purified on a Waters Protein Pak I125 column (7.8 × 300 mm) (Milligen, Milford, MA). Conditions: flow rate: 2.0 ml/min; isocratic with solvent = 80% acetonitrile made to 0.01% TFA. WatPro retention time: Ixori-PVK (PALIPFPRV-NH_2), 6.0 min. Amino acid analysis was carried out under previously reported conditions [20] and used to quantify the peptide and to confirm identity, leading to the following analysis: Ixori-PVK (PALIPFPRV-NH_2): A[1.0], F[1.0], I[1.0], L[1.0], P[2.6], R[1.0], V[1.0]. The identity of the peptide analog was confirmed via MALDI-TOF MS on a Kratos Kompact Probe MALDI-TOF MS machine (Kratos Analytical, Manchester, UK) with the presence of the following molecular ion $[M+H^+]$: Ixori-PVK (PALIPFPRV-NH_2) 1008.63 Da $[M+H^+]$.

Results and discussion

The search for peptidergic hormones in arthropods as tiny as ticks is generally hampered by the small size of the central nervous system that contains low amounts of bioactive substances. In addition, working on ticks provides the researcher with further problems. First of all, and despite their great economic importance, not a single peptide hormone is known from ticks and bioassays to perform conventional neuropeptides purification strategies are not available. This paucity of information was confirmed by a current search for tick neuropeptides in GenBank that only retrieved sequences for two putative salivary secreted neuropeptide-like proteins from the tick *Ixodes pacificus*. Second, the synganglionic tick nervous system does not exhibit well-separated neurohemal release sites as observed in insects, where neurohormones are accumulated and easily accessible for structural elucidation [12]. Having in mind the positive experiences with the analysis of single identified neurons in an insect [18], we decided to perform the following strategy for the identification of putative diuretic hormones. Antisera raised against insect diuretic peptides, namely leucokinin-1 [21] and Pea-periviscerokinin-2 [22], were used to locate neurons in the CNS of *I. ricinus* which may express peptides that are sequence related to the insect kinins and periviscerokinins. Indeed, this procedure yielded several immunoreactive cells, a majority of these neurons were immunostained by both antisera (Fig. 1). Due to their slightly bluish color (Tyndall effect), we succeeded in detecting a number of these neurosecretory neurons in

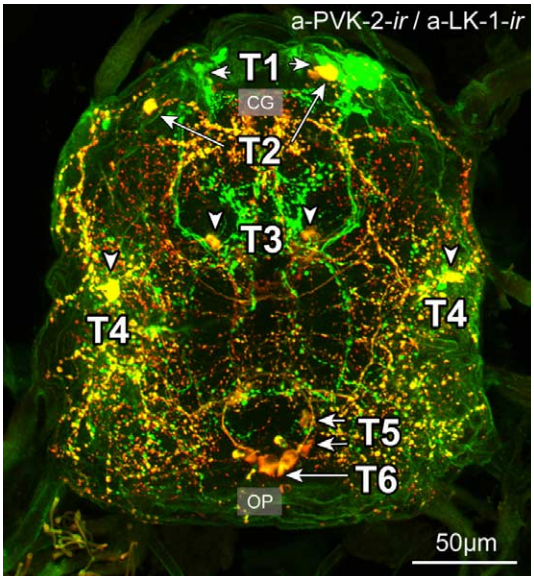

Fig. 1. Double-immunofluorescence staining in whole-mounts of the nervous system from the European hard tick *I. ricinus* by means of an anti-PVK-2 serum (red fluorescence) and an anti-leucokinin-1 serum (green fluorescence). Five cell groups are immunoreactive (T1–T6), four of these groups (T2–T6) and nearly the total network of arborizations throughout the synganglion are colocalized. Two neurons of the cheliceral ganglion (T1: 20–25 µm) contain only leucokinin-immunoreactivity. CG, cheliceral ganglion; OP, opisthosomal ganglion. Scale bar: 50 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

the untreated central nervous system of *I. ricinus*. The most distinctive somata (T1) were found in the cheliceral ganglion where four neurons are immunostained on each side. Two of these neurons (20–25 µm) showed exclusively leucokinin-immunoreactivity and developed extensive and segmentally arrayed arborizations (Fig. 2), resembling the histamine-immunoreactive projections in spiders [23]. In the next step, these large somata were individually dissected and directly prepared for mass spectrometric analysis. Resulting mass spectra showed different ion signals in the mass range of 600–3000 Da with the 1395.69 Da signal

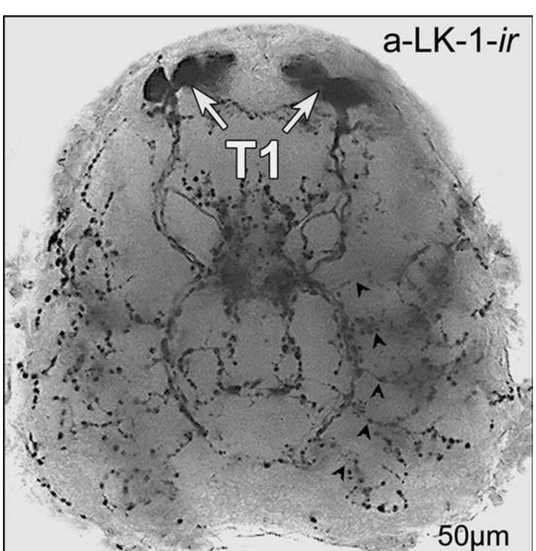

Fig. 2. Microphotograph of leucokinin-like immunoreactivity in the synganglion of *I. ricinus* with extensive and segmentally arrayed arborizations that resemble the histamine-immunoreactive projection in spiders (black arrows). Large anterior neurons (T1) were first isolated and analysed by mass spectrometry (white arrows). Scale bar: 50 µm.

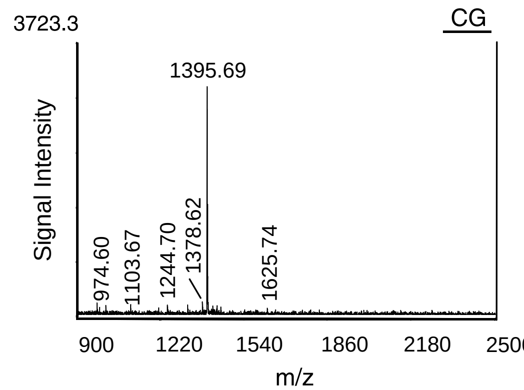

Fig. 3. MALDI-TOF mass spectrum of a T1 neuron from the cheliceral ganglion (CG) of the synganglion from *I. ricinus*. The most abundant ion signal at 1395 Da, as well as further less prominent ions were subjected to fragmentation but did not yield any fragments. Thus, the substances in these T1 neurons are either not fragmenting well or not peptides at all.

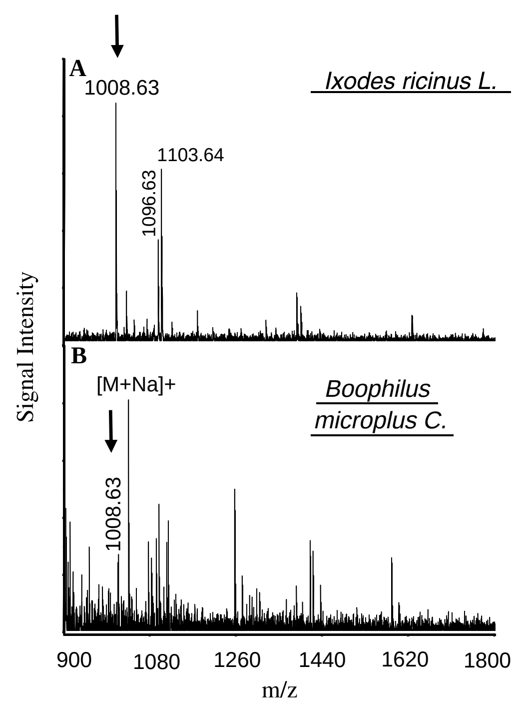

Fig. 4. Representative MALDI-TOF mass spectra of identified homolog cells (T4/T5) (*Boophilus:* cell group) from (A) central European hard tick *I. ricinus* L. and (B) southern cattle tick *B. microplus* C. In both spectra, a mass peak at $[M+H]^+$: 1008.63 Da (arrow) was observed which was subsequently fragmented. Remaining, ion signals differed somewhat between the species.

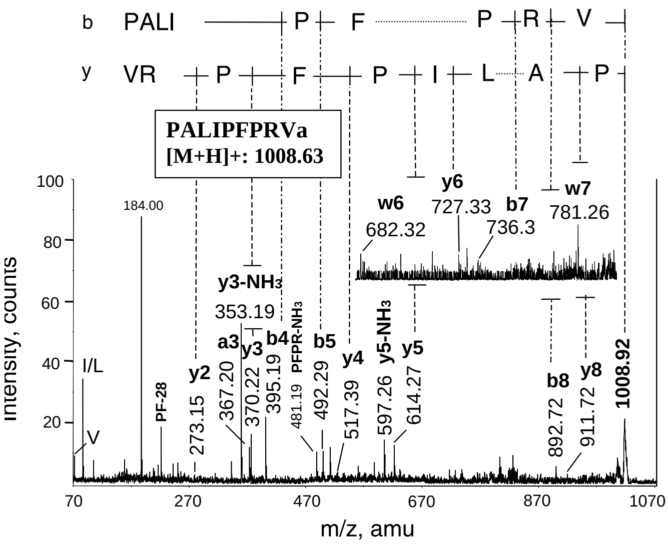

Fig. 5. CID mass spectrum of the peptide at $[M+H]^+$: 1008.63 Da under condition of high gas. The fragments were analysed manually and the resulting sequence is given. A number of *y*-, *a*-, *b*-type, and the *w*-fragment (inset) ions are labelled which not only enabled the complete de novo sequencing but also assignments of Leu/Ile. The identical peptide, named Ixori-PVK, was subsequently sequenced from the other ticks too.

being the most abundant one (Fig. 3). However, no fragment could be obtained from any of these substances, even under conditions of collision-induced dissociation (CID). Control experiments with identified insect neurons verified that the overall signal intensity of the parent ions was sufficient to lead to a number of fragment ions (not shown). Thus, the observed substances in these leucokinin-immunoreactive neurons either do not fragment well or are not peptides at all. Subsequently, neurons of the opisthosomal ganglion (Fig. 1: T5/T6; 10–15 μm) were analyzed using the same technique as described above. Some of these preparations yielded mass spectra with a distinctive ion signal at 1008.63 Da (Fig. 4A). This substance was chosen for fragmentation and produced clear fragments under conditions of low energy fragmentation as well as high energy CID (Fig. 5). The following sequence could be reconstructed from the calculation of mass differences between successive fragments of the different ion series: PA(L/I)(L/I)PFPRV-NH_2. Using the information obtainable from the CID spectra, even the assignment of the L/I ambiguities was possible [14,24] (Fig. 5, inset). The novel peptide is clearly related to the insect periviscerokinins (see Table 1) and is designated as *I. ricinus* periviscerokinin (Ixori-PVK). This is the first successful de novo sequencing of a peptide from single cell preparations of arthropods, and particularly the correct assignment of Leu/Ile from such a tiny sample is an excellent verification of the opportunities associated with modern mass spectrometry. Fragmentations of synthetic Ixori-PVK yielded spectra that are nearly identical with those of native PVKs from single cell preparations.

Homologue cells of another tick species, *B. microplus*, were subjected to mass spectrometric analysis (Figs. 4B and C). *B. microplus*, which transmits several diseases known collectively as 'tick fever,' is a very small tick (roughly 1–2 mm body length). Nevertheless, mass spectra taken from the respective neurosecretory neurons in the opisthosomal ganglion verified the expression of Ixori-PVK in this species as well (not shown).

A screening of the anterior leucokinin-immunoreactive cells of the cheliceral ganglion of *B. microplus* also revealed the abundant ion signal at 1395.69 Da, which altogether indicates the close phylogenetic relationship between the studied tick species.

In summary, the single cell approach, as described in this study, allowed the elucidation of the first peptidergic neurohormone from arachnids. The origin of another peptide recently isolated from 10,000 synganglia of *Amblyomma testindinarium* [25] awaits confirmation since it is very closely related to mammalian hemorphins and may result from contamination due to the impossibility of the complete removal of the esophagus from the synganglion. Ixori-PVK belongs to the periviscerokinin/$CAP2_b$ peptide family, which was previously known only in insects. The effects of periviscerokinins in water balance are well established [26] and it thus seems to be possible that the tick PVK may also regulate diuresis in these blood-feeding arthropods. This function, however, has yet to be established. In the future, the sequence information may aid in the development of mimetic analogs capable of disrupting such critical physiological processes in ticks.

Table 1
Amino acid sequences of members of the periviscerokinin/$CAP2_b$ peptide family

Species name	Peptide name	Peptide sequence	$[M+H]^+$, *m/z*
Insects			
Drosophila melanogaster	Drome-PVK-1/CAP-1	GANMGLYAFPRV-NH_2	1294.67
	Drome-PVK-2/CAP-2	ASGLVAFPRV-NH_2	1015.61
Manduca sexta	Manse-$CAP2_b$	pELYAFPRV-NH_2	976.54
Locusta migratoria	Locmi-PVK-1	AAGLFQFPRV-NH_2	1104.63
Leucophaea maderae	Leuma-PVK-1	GSSGLIPFGRT-NH_2	1090.60
	Leuma-PVK-2	GSSGLISMPRV-NH_2	1102.60
	Leuma-PVK-3	GSSGMIPFPRV-NH_2	1146.61
Acari			
Boophilus microplus	Ixori-PVK	PALIPFPRV-NH_2	1008.63
Ixodes ricinus			

Acknowledgments

The authors thank Renate Winkler and Sabine Kaltofen (Jena, Germany) for providing the European hard tick *I. ricinus* L., Manfred Eckert (Jena, Germany) for the PVK antiserum, Dick R. Nässel (Stockholm, Sweden) for the leukokinin antiserum, and Allison Strey for technical assistance (College Station, TX). This study was supported by a grant from Deutsche Forschungsgemeinschaft (Predel 595/6-1), a Binational Agricultural Research and Development Grant (BARD #IS-3356-02) (R.J.N.), a Collaborative Research Grant (#LST.CLG.979226) from the North Atlantic Treaty Organization (NATO) (R.J.N., G.M.C.), grant from the USDA/DOD DWFP Initiative (#0500-32000-001-01R) (R.J.N.), a grants provided to the Laboratory for Biological Mass Spectrometry at Texas A&M University, College Station: R01 RR019587 NIH and DE-FG02-04ER15520 and by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service (CSREES), and Grant NRI/CSREES 2003-01347 (P.V.P.).

References

[1] United States Department of Agriculture, Agricultural Research Service, <http://www.ars.usda.gov/>.

[2] K.W. Beyenbach, Transport mechanisms of diuresis in Malpighian tubules of insects, J. Exp. Biol. Rev. 206 (2003) 3845–3856.

[3] G.M. Coast, C.S. Garside, Neuropeptide control of fluid balance in insects, Ann. N. Y. Acad. Sci. 1040 (2005) 1–8.

[4] P.V. Pietrantonio, C. Jagge, S. Taneja-Bageshwar, R.J. Nachman, R. Barhoumi, The mosquito *Aedes aegypti* (L.) leucokinin receptor is a multiligand receptor for the three *Aedes* kinins, Insect Mol. Biol. 14 (2005) 55–67.

[5] V.A. Te Brugge, D.A. Schooly, I. Orchard, The biological activity of diuretic factors in *Rhodnius prolixus*, Peptides 23 (2002) 671–681.

[6] V.A. Te Brugge, I. Orchard, Evidence for CRF-like and kinin-like peptides as neurohormones in the blood-feeding bug, *Rhodnius prolixus*, Peptides 23 (2002) 1967–1979.

[7] P.J. Gaines, K.S. Brandt, A.M. Eisele, W.P. Wagner, C.M. Bozic, N. Wisnewski, Analysis of expressed sequence tags from subtracted and unsubtracted *Ctenocephalides felis* hindgut and Malpighian tubule cDNA libraries, Insect Mol. Biol. 11 (2002) 299–306.

[8] O. Tully, D.T. Nolan, A review of the population biology and host–parasite interactions of the sea louse *Lepeophtheirus salmonis* (Copepoda: Caligidae), Parasitol. Rev. 124 (2002) 165–182.

[9] G. Gade, Regulation of intermediary metabolism and water balance of insects by neuropeptides, Annu. Rev. Entomol. 49 (2004) 93–113.

[10] G.M. Coast, The influence of neuropeptides on Malpighian tubule writhing and its significance for excretion, Peptides 19 (1998) 469–480.

[11] C. Wegener, Z. Herbert, M. Eckert, R. Predel, The periviscerokinin (PVK) peptide family in insects: evidence for the inclusion of CAP(2b) as a PVK family member, Pept. Rev. 23 (2002) 605–611.

[12] R. Predel, C. Wegener, W.K. Russell, S.E. Tichy, D.H. Russell, R.J. Nachman, Peptidomics of CNS-associated neurohemal systems of adult *Drosophila melanogaster*: a mass spectrometric survey of peptides from individual flies, J. Comp. Neurol. 474 (2004) 379–392.

[13] E. Pollak, M. Eckert, L. Molnar, R. Predel, Differential sorting and packaging of capa-gene related products in an insect, J. Comp. Neurol. 481 (2005) 84–95.

[14] R.J. Nachman, W.K. Russell, G.M. Coast, D.H. Russell, J.A. Miller, R. Predel, Identification of PVK/CAP2b neuropeptides from single neurohemal organs of the stable fly and horn fly via MALDI-TOF/TOF tandem mass spectrometry, Peptides (in press).

[15] M.D. Adams, S.E. Celniker, R.A. Holt, et al., The genome sequence of *Drosophila melanogaster*, Science 287 (2000) 2185–2195 (over 100 authors).

[16] S.P. Holmes, H. He, A.C. Chen, G.W. Ivie, P.V. Pietrantonio, Cloning and transcriptional expression of a leucokinin-like peptide receptor from the southern cattle tick, *Boophilus microplus* (Acari: Ixodidae), Insect Mol. Biol. 9 (2000) 457–465.

[17] S.P. Holmes, R. Barhoumi, R.J. Nachman, P.V. Pietrantonio, Functional analysis of a G protein-coupled receptor from the southern cattle tick *Boophilus microplus* (Acari: Ixodidae) identifies it as the first arthropod myokinin receptor, Insect Mol. Biol. 12 (2003) 27–38.

[18] S. Neupert, R. Predel, Mass spectrometric analysis of single identified neurons of an insect, Biochem. Biophys. Res. Commun. 327 (2005) 640–645.

[19] M. Eckert, Z. Herbert, E. Pollak, L. Molnar, R. Predel, Identical cellular distribution of all abundant neuropeptides in the major abdominal neurohemal system of an insect (*Periplaneta americana*), J. Comp. Neurol. 452 (2002) 264–275.

[20] R.J. Nachman, R.E. Isaac, G.M. Coast, G.M. Holman, Aib-containing analogues of the insect kinin neuropeptide family demonstrate resistance to an insect angiotensin-converting enzyme and potent diuretic activity, Peptides 18 (1997) 53–57.

[21] G.M. Holman, B.J. Cook, R.J. Nachman, Primary structure, and synthesis of two additional neuropeptides from *Leucophaea maderae*: members of a new family of cephalomyotropins, Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 84 (1986) 271–276.

[22] R. Predel, J. Rapus, M. Eckert, G.M. Holman, R.J. Nachman, Y. Wang, H. Penzlin, Isolation of periviscerokinin-2 from the abdominal perisympathetic organs of the American cockroach, *Periplaneta americana*, Peptides 19 (1998) 801–809.

[23] A. Schmid, C. Becherer, Distribution of histamine in the CNS of different spiders, Microsc. Res. Tech. 44 (1999) 81–93.

[24] R.J. Nachman, W.K. Russell, G.M. Coast, D.H. Russell, R. Predel, Mass spectrometric assignment of Leu/Ile in neuropeptides from single neurohemal organ preparations of insects, Peptides 26 (2005) 2151–2156.

[25] J.G. Liang, J. Zhang, R. Lai, H.H. Rees, An opioid peptide from synganglia of the tick, *Amblyomma testindinarium*, Peptides 26 (2005) 603–606.

[26] C. Wegener, D. Linde, M. Eckert, Periviscerokinins in cockroaches: release, localization, and taxon-specific action on the hyperneural muscle, Gen. Comp. Endocrinol. 121 (2001) 1–12.